

Characterization of Biological Fallout Particles of Cleanrooms to Measure Spacecraft Cleanliness

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NASA cleanrooms are humidity, temperature-regulated, and particle contaminant-controlled environments used for spacecraft assembly. Microorganisms, which are crucial contaminants shed from humans and transported through the environment, can adapt to the oligotrophic cleanroom conditions. These biogenic particles need to be identified to extend our knowledge of biological contamination for future NASA mission use. Here, we devised an aluminum coupon with polycarbonate filter setup to collect fallout particles from an ISO 5 and an ISO 7 cleanroom. We demonstrated the presence of microbial particles, as standalone entities and associated with inert particles in cleanroom environments. The contamination was confirmed as biogenic in virtue of estimating the elemental composition of individual and aggregated particles using an epifluorescence and Field Emission Scanning Electron Microscopy (FESEM) coupled with an energy dispersive X-ray analysis (EDX). Epifluorescence microscopy showed that the particles were stained less (25%) with SYTO 9 and more (75%) with propidium iodide, suggesting that the suspected biogenic particles are membrane-compromised. Remarkably, biological particles such as bacteria and fungal spores/hyphae were observed at sizes ranging from 2 to 70 μm , with abundant C and O elemental signatures and essential tracers (Na, Mg, K, Ca and Cl). Inert particles (2 to 500 μm) that were characterized included compositions such as silica (SiO_2), aluminosilicates (Al, Si), and carbonaceous fibers. Furthermore, we designed and illustrated a unique correlative epifluorescence and FE-SEM method to strengthen this approach by acquiring the same biogenic particles in the same filter coupon for a one-to-one comparison. Interestingly, the SYTO 9 stained particles showed abundant C and O elemental signature implying that they are viable microbial particles. Constant evaluation of cleanroom biological and inert particles will help to characterize biological contamination for future NASA missions (e.g. Mars 2020).

Nomenclature

ANOVA	=	Analysis Of Variance
BiSKits	=	Biological Sampling Kits
EDX	=	Energy Dispersive X-Ray Analysis
FESEM	=	Field Emission Scanning Electron Microscopy
ISO	=	International Organization for Standardization
JPL	=	Jet Propulsion Laboratory
NASA	=	National Aeronautics and Space Administration
PC	=	Polycarbonate
PI	=	Propidium Iodide

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I. Introduction

Microorganisms, either airborne or human associated, and non-biological particulates are two major contaminants of concern present in certified cleanroom environments such as spacecraft assembly facilities, intensive care units, pharmaceutical, and medical industry manufacturing units^{1, 2}. The contaminants include (i) biogenic particles such as viruses, pollen, fungi, and bacteria; (ii) geogenic particles such as silica, calcium rich particles; and (iii) anthropogenic particles such as industrial particles and carbonaceous particles³. These particles range from 100 nm to 1,000 μm and have been found to play a critical role in the health and comfort of humans, and the safeguarding of the closed and built-in environment. The reduction of airborne particulates and maintenance of a cleanroom environment are of significant importance in the fabrication and assembly of many industrial products, including electronics, pharmaceuticals, medical devices, and spacecraft hardware components. Terrestrial microbial particle contaminants can potentially hitchhike on spacecraft and impact the scientific integrity of extraterrestrial life-detection experiments⁴⁻⁶. It is therefore essential to understand microbial viability, size distribution, and chemical composition of fallout particles associated with the spacecraft assembly cleanrooms where spacecraft are assembled. Such an approach will help to understand whether these particles are of Earth origin and might be useful to discriminate from authentic extraterrestrial biosignatures. Consequently, such particle characterizations may also be applied as a “source-tracking” tool that can influence how spacecraft are constructed and assembled under stringent quality-controlled cleanroom environment⁷.

National Aeronautics and Space Administration (NASA) spacecraft assembly cleanrooms are known to be extremely clean by way of controlled airflow with filter circulation, controlled temperature and humidity, and rigorous cleaning. In addition, routine counts of simple particles are conducted to monitor the cleanroom in conjunction with a detailed particulate cleanroom characterization necessary to certify the cleanroom. This environment may contain microbial particles that are associated with dust particles, and it is of the highest interest to depict the microbe to particle distribution. Bacterial migration with an attached dust particles is a natural phenomenon that is promoted by atmospheric events⁸. Cleanroom microbial communities have been frequently monitored and investigated by culture-dependent and advance next-generation sequencing methods in an attempt to study the microbial burden and their possible distribution effect on ecosystem^{6, 9, 10}. However, only a few studies have investigated the influences of dust to the culturable bacterial communities distribution ratio of the cleanroom air samples that are limited to only size particle distribution and bacterial identification¹⁰. Furthermore, to the extent of our knowledge, there is no direct evidence and evaluation on the bacterial viability associated with dust particles. It is well known that the nucleic acid based fluorescence imaging technique coupled with the computer assisted automated particle analysis using ImageJ can answer the questions on bacterial viability and distribution ratio of particles that associated with bacteria^{11, 12}. The use of the fluorescence dye propidium iodide (PI) and SYTO9 allows for a more accurate estimation of both free and dust-associated viable and total microorganisms^{8, 13, 14}. Furthermore, characterization of individual particles using Field Emission Scanning electron microscopy (FESEM) coupled with energy-dispersed X-ray analysis (EDX) provide useful information on size, shape, elemental composition of particles and give us a enhanced perception about the origin of particles³.

The objective of this study was to establish a creditable estimate of airborne viable microorganisms that are associated with or free from dust particles of various sizes collected from Jet Propulsion Laboratory (JPL) cleanrooms. Here we demonstrate the relative microbial abundance and viability associated with JPL cleanroom dust particles, which were obtained with a culture-independent approach. The methodology of epi-fluorescence microscopy techniques coupled with fluorescent staining and computer assisted image processing was followed to identify the nature of live/dead microbial particles without traditional microbiology cultivation. Furthermore, we detect the elemental composition and morphological characterization of the particles by Field Emission Scanning Electron Microscopy (FESEM) coupled with EDX. These studies are essential inputs for a reliable biological contamination transport models that help foresee potential sample cross-contamination threats during spacecraft assembly for present and future NASA missions landing on other celestial bodies.

II. Materials and Methods

A. Fallout dust particles:

The fallout sample-collection aluminum witness coupons were assembled with sterile 0.2 μm pore size polycarbonated¹⁵ membrane filters as shown in Figure 1A. The fallout coupons were deployed in the JPL-cleanrooms (ISO 5 and ISO 7) for a two-week period for four consecutive time points to obtain sufficient amount of fallout particles. Collected coupon samples were transferred to the lab, then coupon cassettes were carefully disassembled and the filters were removed with sterilized tweezers and mounted onto a borosilicate glass Buchner

A



(A) 2D cartoon illustration of the aluminum coupon holder depicting the procedure for assembly of the coupon with PC filter to deploy in the JPL cleanrooms. Left panel: 1) Two 0.9" holes on the top portion of the aluminum coupon holder were designed to hold yet maximize the exposure of the PC filter. Three small holes at end of the holder was used to assemble the top and bottom portions with screws. The dimension and placing of the holes are shown in right panel; 2) sterilized 0.2 μm -pore size PC membrane filters; and (3) Bottom aluminum plate designed to hold the PC filters.

B. Epi-fluorescence imaging and ImageJ analysis:

C. Calculation of particle count and statistical analysis:

3

$$P_{\text{bio+inert}} = (P_R + P_G) - P_T \quad (1)$$

P – Fallout particles

P_T – Total count of fluorescence stained particles from the selected PC filter

P_R – Red fluorescence stained particles counts from the selected PC filter

P_G – Green fluorescence stained particles counts from the selected PC filter

Furthermore, to assess (either green or red fluorescence stained) fallout particles from the cleanroom that have no association, we derived following equation;

$$P_{\text{free}} = P_T - P_{\text{bio+inert}} \quad (2)$$

$$P_{G_free} = P_{\text{free}} * (P_G / (P_R + P_G)) \quad (3)$$

$$P_{R_free} = P_{\text{free}} * (P_R / (P_R + P_G)) \quad (4)$$

P_{G_free} – Green fluorescence stained particles counts freely available

P_{R_free} – Red fluorescence stained particles counts freely available

Scatter plot and bar graph of particle count were plotted using Prism (version 7). Significance ($p < 0.05$) between groups was tested by a one-way analysis of variance (ANOVA) using Prism.

D. Electron microscopy:

A FESEM, equipped with an EDX analyzer (FEI Sirion, FEI Co., OR), was used for examination of biological particle and its association with inert particles in the collected fallout filter coupons (Figure 1B). The PC filter coupons were mounted on carbon stubs for carbon coating. A very thin film of carbon (C) was deposited on the surface of each sample using a vacuum coating unit (Leica EM ACE600, USA). EDX analysis is based on analysis of the characteristic X rays emitted when an electron beam is incident on a sample. Unfortunately, the spatial resolution obtainable with EDX analysis is at best about 1 μm . The acceleration voltage used to analyze samples was 10 to 20 kV, a beam current of 40 – 50 μA and a detector 6 – 10 mm away from the sample to be analyzed. Similar settings were maintained when different models or SEM instruments were used. The EDX analysis was carried out at each analysis point and the element present were both qualitatively and quantitatively measured. The weight percentage of each element present in the spectrum was identified.

III. Results

A. Visualization of individual fallout particles from JPL-cleanroom:

Table 1. Biological fallout coupon deployment dates and sampling locations of the JPL cleanrooms. 3 locations from ISO 7 designated as ‘a’, ‘b’ and ‘c’, and 1 location from ISO 5 as ‘d’.

Coupon deployment and collection date	I - Deployment (08/08/16 – 08/22/16)				II - Deployment (08/22/16 – 09/05/16)				III - Deployment (09/05/16 – 09/19/16)				IV - Deployment (09/19/16 – 10/03/16)			
	ISO 7		ISO 5		ISO 7		ISO 5		ISO 7		ISO 5		ISO 7		ISO 5	
	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
Epi - 1	1	5	9	13	17	21	25	29	33	37	41	45	49	53	57	61
Epi - 2	2	6	10	14	18	22	26	30	34	38	42	46	50	54	58	62
Epi - 3	3	7	11	15	19	23	27	31	35	39	43	47	51	55	59	63
Epi - 4	4	8	12	16	20	24	28	32	36	40	44	48	52	56	60	64

In total, 64 polycarbonate filters were deployed to collect fallout particles, imaged, and microbial cells and inert particles were counted using the ImageJ based automated particles counter (Table 1). Of the 64 PC filter, 48 filters were deployed in an ISO 7 cleanroom, and 16 filters were deployed in an ISO 5 cleanroom over 2 weeks for three consecutive sampling events. Figure 2A shows the PC filter global epifluorescent microscopy image of the collected fallout particles from the ISO 7 cleanroom at JPL. Under blue excitation rays, BacLight fluorescently labeled biological cells emit green, yellow, and red fluorescence. However, even inert dust particles with mineral particles were emitting yellow and red colors. Frequently, we observed more individual particles stained either red, likely to be dead or green viable microbial cells, with fewer inert dust particles. An example of a live microbial particle (distinguished

with the PI stain), a tubular fungal hypha, is shown in Figure 2A-B. Notably, we could frequently detect standalone live and dead biological and mineral like particles as shown in Figure 2C. Furthermore, the negative control PC filters showed that there is no potential contamination during assembly and processing (data not shown)¹⁴.

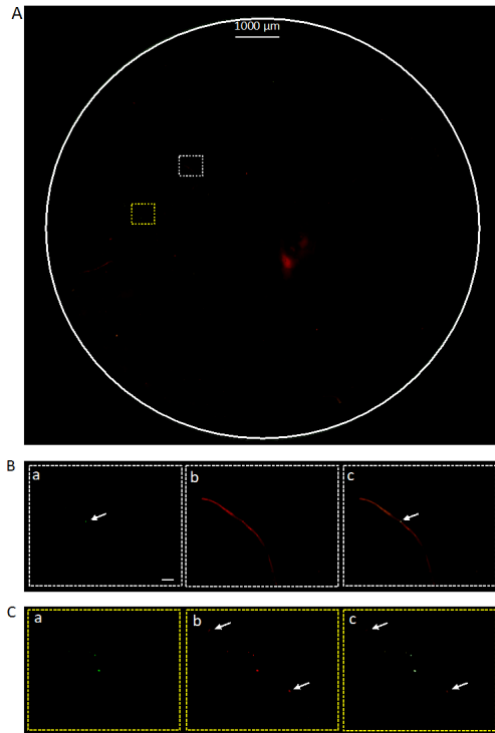


Figure 2. Epi-fluorescence imaging of microbial cells on fallout particles collected from ISO7 cleanroom. (A) Stained PC filters were removed aseptically from the filter funnel and placed back on the coupon, examined using epi-fluorescence microscopy and the images were subsequently acquired and stitched to get single global image of PC filter. a) The epi-fluorescent image of the global fallout PC filter collected from JPL ISO 7 cleanroom

(B) Selected single fluorescence image of (A). Shown are SYTO9 signal from live bacterial cell (a), PI signal from fungal hyphae like structure (b) and an composite image of (a) and (b) (c). Scale bar represents 100 µm; white arrow highlighted that the live bacterial cell attached to the fungal hyphae

(C) Selected single fluorescence image of (A). Shown are SYTO9 signal from live bacterial cell (a), PI signal from dead cell or inert particles (b) and a composite image of (a) and (b) (c). Scale bar represents 100 µm; white arrow highlighted only PI fluorescence particles.

Scatter plots were generated to ensure the distribution of ISO 7 cleanroom fallout particle count observed. The distribution of the biological particles had no significant differences within and between PC filters (Figure 3A). Of the 28 PC membranes from 48 filters, the average fallout particles abundance was 2.6×10^3 per sample collected from three different locations within the ISO -7 JPL cleanroom from August 8, 2016 to October 03, 2016. Furthermore, growth and correlative microscopy techniques were utilized to visualize and count the particle of same filter and same particles (data not shown and not included in this study analysis¹⁶. Red fluorescent stained particles (P_{R_free}) that were either dead biological or inert particles constituted 77%, green fluorescent particles (P_{G_free}) that were viable microorganisms comprised of 7%, and the microorganisms associated particles ($P_{bio+inert}$) were only 16% of the total counted particles (Figure 3A-B). In total, the biological contamination among total particles that were captured through fallout approach was only 23%.

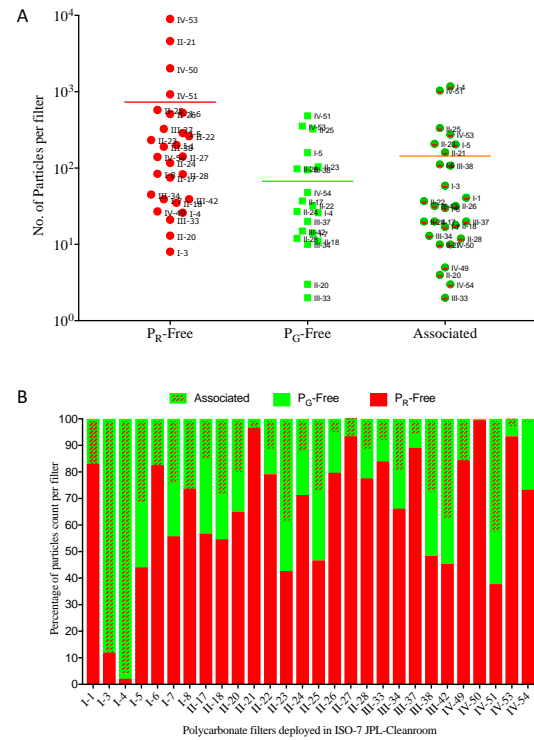


Figure 3. Enumerating the fallout particles using ImageJ:

(A) Scatter plot of the fallout particles count collected from JPL ISO 7 cleanroom. Each dot in a column represents the number of particles counted on each polycarbonate filters using ImageJ software. The particles were calculated using the equation (1 - 4) to split into free and associated particles.

Red dot: likely to be dead bacterial/fungal hyphae or inert dust particles, Green square: likely to be live microbial particles, and red/green dot: likely to be microbial particles associated with inert dust particles. No statistically significant differences in particles count were observed amongst particle types and between different sampling locations (1 way- ANOVA, $p > 0.5$).

(B) Bar graph showing the percentage of particle counts collected from JPL ISO 7 cleanroom. The red bar represents the percentage of particles that are likely to be dead bacterial/fungal hyphae or inert dust particles. The green bar represents the percentage of particles likely to be viable microbial particles. The red and green mixed bar represents the percentage of particles likely to be microbial particles associated with inert dust particles. Note: I – Deployment1, II – Deployment2, III – Deployment3 and IV – Deployment4 at ISO 7 cleanroom.

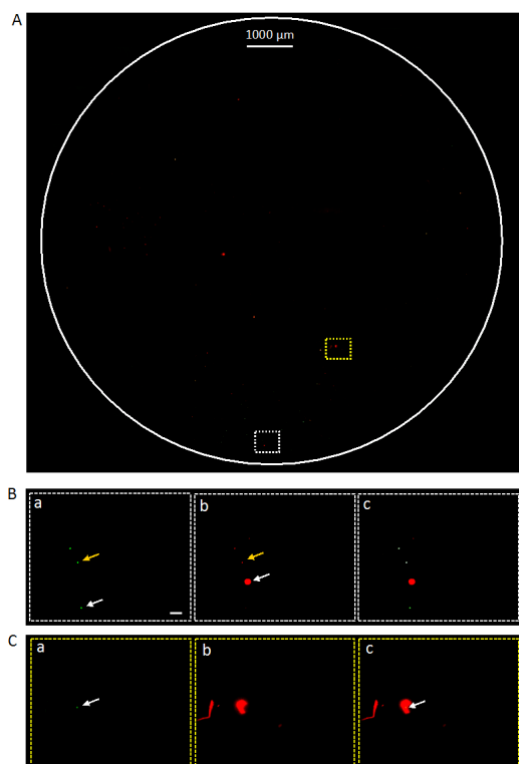


Figure 4. Epi-fluorescence imaging of microbial cells on fallout particles collected from ISO5 cleanroom. (A) Stained PC filters were removed aseptically from the filter funnel and placed back on the coupon, examined using epi-fluorescence microscopy, and subsequently the acquired images were stitched to get single global image of PC filter. a) Shown is the epi-fluorescent image of the global fallout PC filter collected from JPL ISO 5 cleanroom (B) Selected single fluorescence image of (A). Shown are SYTO9 fluorescence signal from live bacterial cell (a), PI fluorescence signal from dead bacterial cell or inert particles (b) and a composite image of (a) and (b) (c). Scale bar represents 100 µm; white arrow highlighted standalone biological particles and yellow arrow highlighted the association biological particles. (C) Selected single fluorescence image of (A). Shown is a SYTO9 fluorescence signal from live bacterial cell (a), PI fluorescence signal from dead cell or inert particles (b) and a composite image of (a) and (b) (c). Scale bar represents 100 µm; white arrow highlighted standalone live bacterial particles associated with inert particles.

Fluorescence stained global stitched PC filter image of fallout particles collected from ISO 5 cleanroom are depicted (Figure 4A). Often, we observed dead microbial cells (PI stained particles) and fewer live cells (SYTO9 stained particles). Remarkably, we could observe free floating live and dead microbial particles (Figure 4B) in the ISO5 cleanroom location, but in general no significant difference in the distribution of particles. Furthermore, we also detected live bacterial cells attached to mineral like particles shown in Figure 4C. The distribution of particle

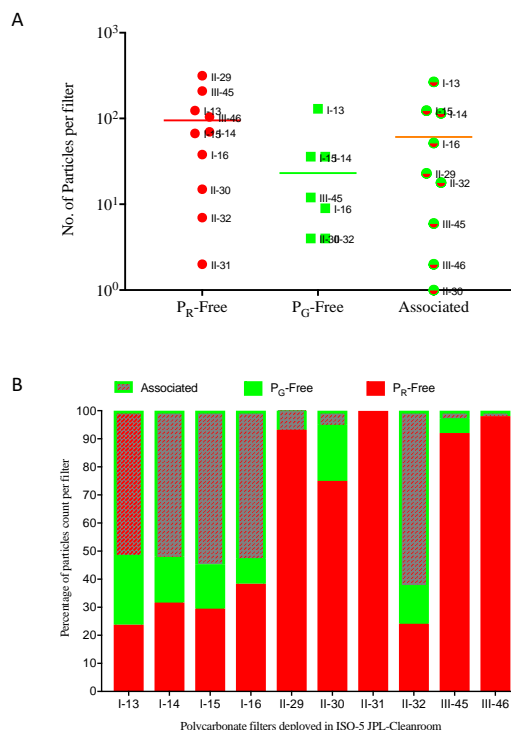


Figure 5. Enumerating the fallout particles of JPL ISO 5 using epi-fluorescence and ImageJ: (A) Scatter plot of the fallout particles count collected from JPL ISO 5 cleanroom. Each dot in a column represents the number of particles counted on each polycarbonate filters using ImageJ software. The particles were calculated using the equation (1 - 4) to split into free and associated particles.

Red dot: likely to be dead bacterial/fungal hyphae or inert dust particles, Green square: likely to be live microbial particles and red/green dot: likely to be microbial particles associated with inert dust particles. No statistically significant differences in particles count were observed amongst particle types and between different sampling locations (1 way- ANOVA, $p > 0.5$).

(B) Bar graph showing the percentage of particle counts collected from JPL ISO 5 cleanroom. Red bar represents the percentage of particles that are likely to be dead bacterial/fungal hyphae or inert dust particles. The green bar represents the percentage of particles are likely to be live microbial particles. The red and green mixed bar represents the percentage of particles are likely to be microbial particles associated with inert dust particles.

Note: I – Deployment1, II – Deployment2, III – Deployment3 and IV – Deployment4 at ISO5 clean room.

with and without biological materials associated with ISO 5 is shown in Figure 5A. Of the 10 PC membrane from 16 filter the average fallout particles abundance was 1.8×10^2 per sample collected from ISO -5 JPL cleanroom from August 8, 2016 to October 03, 2016. Other filters were used for advance correlative microscopy techniques, data not shown and not included in this study analysis¹⁶. Red fluorescent stained particles (P_{R_free}) that were either dead biological or inert particles constituted 53%, green fluorescent particles (P_{G_free}) that were viable microorganisms comprised of 13% (twice more than in ISO 7 cleanroom), and the microorganisms associated particles ($P_{bio+inert}$) were 34% of the total counted particles. In total, the biological contamination among total particles that were captured through fallout approach was only 47% (Figure 5B).

B. Elemental characterization of particles collected from JPL-cleanrooms:

Fallout particles structural morphology and size distribution were determined using FESEM as shown in (Figure 1B). The particles analyzed in the study were mostly ranging from 2 to 20 μm and different groups of particles display variable morphologies. A typical SEM microscopy image of carbonaceous particles that was dominated by C and O is shown in Figure 6A-B. The elemental composition and morphology of the particles are characterized during EDX analysis. Biological particles that are both viable and non-viable contained minor amounts of Na, Mg, K, Al, Si and Ca (usually <10% of relative element intensity), which are essential tracers present in plants³. As an example, an irregular shaped aggregated organic particle is shown in Figure 6C which was abundant with C and O while other essential tracers (Na, Mg, Al, Si, S, Cl and Ca) are observed in minor amounts. Microorganisms and fragments of all varieties of living matter such as fungal spores, pollen and plant debris possessed these EDX patterns.

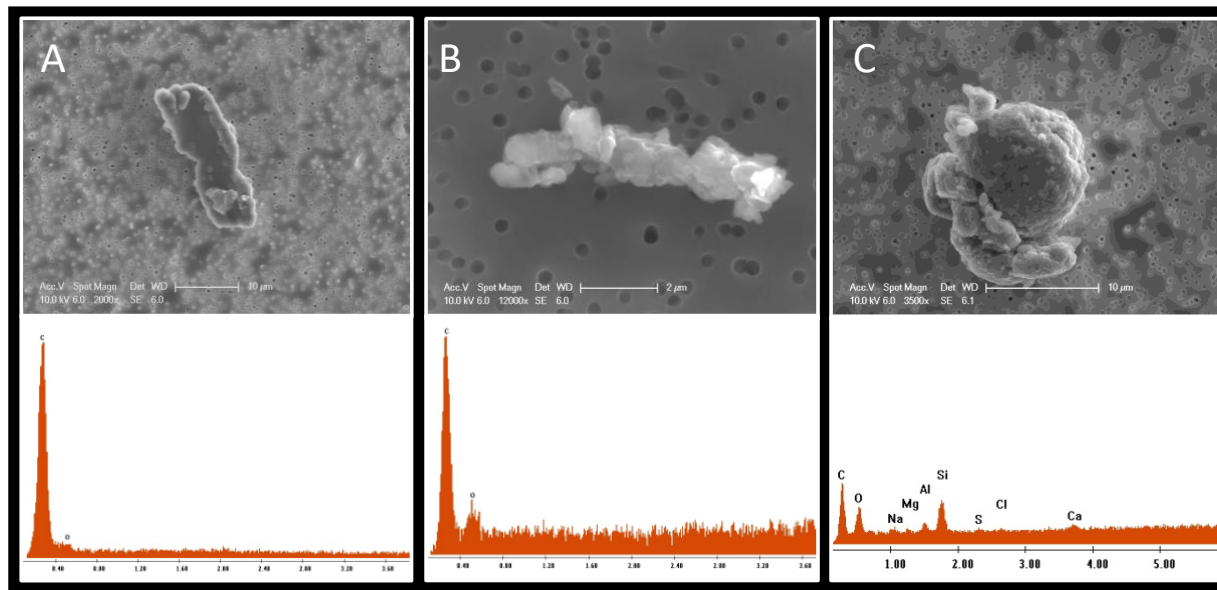


Figure 6. Visualization and elemental composition analysis of biological fallout particles using FESEM coupled with EDAX. Fallout coupons collected from JPL cleanroom were carbon coated, visualized by FESEM, and elemental composition analyzed by EDAX. (A) Image (x2000) of a carbonaceous particle with irregular shape; (B) Image (x12000) of a carbonaceous particle with irregular chains of rod like structures; and (C) Image (x3500) of a grossular particle with irregular shape.

IV. Discussion

Cleanrooms are designed to control environmental factors including containing airborne particles, airflow patterns, temperature and humidity, and containment of hazardous aerosols. These rooms are used to manufacture sensitive products such as electronics, medical products, and aerospace components. The presence of dust particles > 0.5 μm in size would include fibers, hair, and other biological particles that also affect the adsorption and adhesion of other dust particles on a surface by either coagulation or electrostatic charging^{17, 18}. The JPL planetary protection group's highest priority is to assess the cleanliness of spacecraft assembly to prevent the contamination of current and future spacecraft missions^{19, 20}. Moreover, the quantitative assessment of microorganisms associated with fallout particles and its variance by particle size distribution within JPL cleanrooms is largely unknown. Prior studies have

been mostly focused on microbial surveillance associated with dust particle using various culture-dependent and molecular biological techniques. Exploration of the fallout microbial particles associated with cleanroom environment would further contribute to the indoor bioaerosol scientific research. This will benefit the development of suitable spacecraft as well as basic and applied research in cleanroom environments.

Since the Viking missions to Mars in the 1970s, the majority of studies have utilized standardized sampling devices for collecting samples from spacecraft assembly facility for estimating microbial burden²¹. These procedures specifically utilize cotton swabs, wipes, biological sampling kits (BiSKits), and other air samplers for collection of particulates to estimate microbial burden/diversity in dust. However, this approach could not determine the microbial abundance and viability associated with dust and non-dust particles²²⁻²⁴. The main goal of this study was to enumerate the distribution of viable microorganisms in association with the cleanroom dust particle.

We proposed a new polycarbonate filter based aluminum witness coupon setup to collect and to evaluate particle dispersal of spacecraft assembly facilities (Figure 1A). PC membrane filters (the pores can be as small as 200 nm) have been most widely used for direct bacterial or dust particle count and are also suitable for surface observation of particles using various microscopy techniques^{25, 26}. Notably, visualization of fluorescence stained cell or particles using a PC membrane filter plays a crucial role in reducing fluorescence dye background and auto-fluorescence²⁶. Furthermore, utilization of live/dead staining using SYTO9 and PI offers a rapid and reliable method to assess bacterial viability. However, both dyes can unequally stain either the same particles or cells, particularly dead gram negative bacteria that shows 18-fold stronger SYTO9 signal than PI. Here, the accurate estimation of viable cells was not disturbed because the two channels were evaluated distinctly using ImageJ¹⁴. Our observation indicated that the bacterial abundance and distribution of the viable and total particles, varied among cleanrooms. There is a possibility that the detected bacterial cells were influenced somewhat by contaminants from local sources such as human skin, clothing and personal care products, or outdoor particles such as building material fragments⁷. The average total number of cleanroom contaminants (6.7×10^1) of viable, freely floating microbial particles from local sources, such as human skin associated microbes, are comparatively lower than averaged total dead microbial particles in fallout dust (7.33×10^3). The result suggests that the dust particles in the samples combined with the cleanroom extreme conditions might inhibit the viability of microbial cell counts and their ability to survive⁸.

The particles analyzed in this study were mostly of large sizes with diameter ranging from 2 to 100 μm . An irregular carbon particle (>90% of C and O) observed in our study might be from outdoors particles (Figure 6). Cleanroom fallout particles were clearly dominated by biogenic particles with minor elements, and few carbonaceous particles which could originate from industrial biological processes. Subsequently, imaging the same particles seen on the PC filter using correlative epi-fluorescence and FESEM-EDX was possible, which supports and provides better understanding of the visualization of a live microorganism and its association with dust particles^{16, 27}. The outcome of this study will gain useful information for effective countermeasures to maintain the cleanliness to prevent biological contamination of current and future planetary protection sensitive missions.

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